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der Vetsuisse-Fakultät Universität Zürich

The Significance of gE Domains of Bovine Herpes Virus Type 1 as Revealed by High Resolution Electron Microscopy

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SUMMARY

Bovine Herpes Virus 1 envelopment was shown to follow 2 pathways. Nuclear envelopment involves budding at the inner nuclear membrane, transportation via the endoplasmic reticulum into Golgi cisternae and packaging into vacuoles. For cytoplasmic envelopment, capsids escape the nucleus via impaired nuclear pores and approach cell membranes where they bud at Golgi membranes. Glycoprotein E (gE) is involved in envelopment, and facilitates cell to cell spread. Site of action and mode of function are not well understood. Here the significance of gE is investigated using mutants of which gE or parts thereof was deleted or replaced. High resolution electron microscopy of cells infected with gE deletion mutants revealed that i) capsids accumulated at budding sites at nuclear membranes, ii) polymorphism and number of capsids per virion was enhanced, iii) virions accumulated within Golgi cisternae, iv) solid capsid-tegument aggregates were formed within nuclei, and released by budding or possibly via impaired nuclear pores, and v) tegument and capsids accumulated adjacent to Golgi membranes. These findings indicate that the entire gE is involved in budding at the nuclear membrane and at Golgi membranes facilitating optimal acquisition of tegument and formation of optimally sized virions, and in fission of Golgi membranes to form transport vacuoles. The insertion of spikes into vacuolar membranes suggests that gE may have pilot functions in transportation towards the cell periphery.

INTRODUCTION

Herpesvirus envelopment has been controversially discussed for decades (see ref. (Homman-Loudiyi, Hultenby et al. 2003). One reason for this was probably the application of inadequate tools to investigate the rapid process of envelope formation initiated by capsids approaching cell membranes. Employment of cryofixation followed by freeze-substitution that yields both high spatial and high temporal resolution shed light on envelopment of bovine herpesvirus 1 (BoHV-1) (Wild, Schraner et al. 2002; Wild, Engels et al. 2005) and herpes simplex virus 1 (HSV-1) (Leuzinger, Ziegler et al. 2005). Capsids of these members of the α-herpesvirus family exit the nucleus either by budding at the inner nuclear membrane or via impaired nuclear pores (Wild, Schraner et al. 2002; Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005) as was suggested for simian agent 8 (Borchers and Oezel 1993). There is strong evidence that virions originating by budding at nuclear membranes are transported via RER cisternae (Schwartz and Roizman 1969; Stannard, Himmelhoch et al. 1996) directly into Golgi cisternae where they are packaged into transport vacuoles (Wild, Schraner et al. 2002). This route involving budding at the inner nuclear membrane, intraluminal transportation, and packaging in Golgi cisternae was designated *nuclear envelopment* (Leuzinger, Ziegler et al. 2005). Capsids escaping the nucleus via impaired nuclear pores approach intracellular membranes, particularly those of the Golgi complex, where they start to bud. Budding at narrow Golgi cisternae results in a concentric, double membranous compartment, the inner being the virion with its envelope, the outer the membrane of the transport vacuole. This process is designated wrapping. Budding at dilated Golgi cisternae or Golgi derived vacuoles results in virions within cisternae or vacuoles. These virions cannot be distinguished from those deriving from packaging unless the budding process is captured. Virions originating by budding at the outer nuclear membrane or at RER membranes need to be transported to Golgi cisternae for packaging like the virions originating by budding at the inner nuclear membrane (Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005). The route involving release of capsids via impaired nuclear pores, and budding at the outer nuclear membrane, RER membranes or Golgi membranes was designated *cytoplasmic envelopment*. These data and many previously published results on herpesvirus envelopment e.g. (Darlington and Moss 1968; Schwartz and Roizman 1969; Johnson and Spear 1982; Torrisi, Di Lazzaro et al. 1992; Stannard, Himmelhoch et al. 1996) clearly contradict the currently propagated view of de-envelopment of perinuclear virions by fusion of the envelope with the outer nuclear

membrane whereby capsid and tegument are released into the cytoplasm (Granzow, Klupp et al. 2004).

The duality of nuclear exit requires signaling and controlling mechanisms acting within the nucleus and/or at the nuclear membrane. Budding at cellular membranes including inner and outer nuclear membrane, RER membranes and Golgi membranes may be initiated and controlled by the same factor(s) present on capsids, tegument and/or on the membrane involved in budding. Many proteins, which are involved in envelopment, have been localized on the nuclear surface and/or on virions within the perinuclear space, e.g. U_L31, U_L34 and U_S3 (Reynolds, Wills et al. 2002; Granzow, Klupp et al. 2004), U_L11 (Baines, Jacob et al. 1995), glycoprotein K (gK) (Rajcani and Kudelova 1998), glycoproteins E and I (gE and gI) (Wang, Gershon et al. 2001). gE and other glycoproteins have been shown to be present at the Golgi complex (review ref. (Mettenleiter 2004)

The significance of gE is not well understood. Virus yield of BoHV-1 ΔgE is not affected (Shaw, Braun et al. 2000), that of HSV-1 ΔgE is slightly reduced (Balan, Davis-Poynter et al. 1994). One of its main function is promoting cell-to-cell spread (Balan, Davis-Poynter et al. 1994; Dingwell, Brunetti et al. 1994; Dingwell, Doering et al. 1995; Dingwell and Johnson 1998; Collins and Johnson 2003; Mettenleiter 2004; Ch'ng and Enquist 2005). Deletion of gE did not affect envelopment (Farnsworth, Goldsmith et al. 2003). Deletion of gE in combination with gD and/or gI in HSV-1 (Farnsworth, Goldsmith et al. 2003) or in combination with gM or gI and gM in pseudorabies virus (PRV) (Brack, Klupp et al. 2000), however, resulted in profound inhibition of cytoplasmic envelopment. In PRV infected cells gE was localized at the trans Golgi network (Zhu, Hao et al. 1996; Alconada, Bauer et al. 1998; Johnson, Webb et al. 2001; McMillan and Johnson 2001). The localization of gE at both sites the nuclear envelope and the trans Golgi network prompted us to investigate whether or not gE per se plays a role along the developmental pathways. High resolution electron microscopy of MDBK cells infected with diverse gE deletion mutants of BoHV-1 revealed evidence that gE is involved in both nuclear and cytoplasmic envelopment and that it facilitates virus transportation from the trans Golgi site towards the plasma membrane.

MATERIAL AND METHODS

Cells and Viruses

Madin Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle's medium (D'MEM: Gibco, Bethesda, MD, USA) supplemented with 10% fetal calf serum (Gibco) at 37°C and 5% CO_2. Wild-type (wt) BoHV-1 (strain Jura and LAM) and recombinant BoHV-1 were propagated in MDBK cells.

Glycoprotein E Deletion Mutants

Three different gE deletion mutants (Fig. 1). were used: ΔgE from BoHV-1 strain LAM (van Engelenburg, Kaashoek et al. 1994),

LAM gE-ΔCT and LAM gE-ΔTMCT (Tyborowska, Bienkowska-Szewczyk et al. 2000; Rychlowski, Rijsewijk et al. 2001).

A recombinant virus, rCS124 from BoHV-1 strain Jura, in which the ectodomain of gE was replaced with the ectodomain of gB of ovine herpesvirus-2 (Senn, unpublished data)

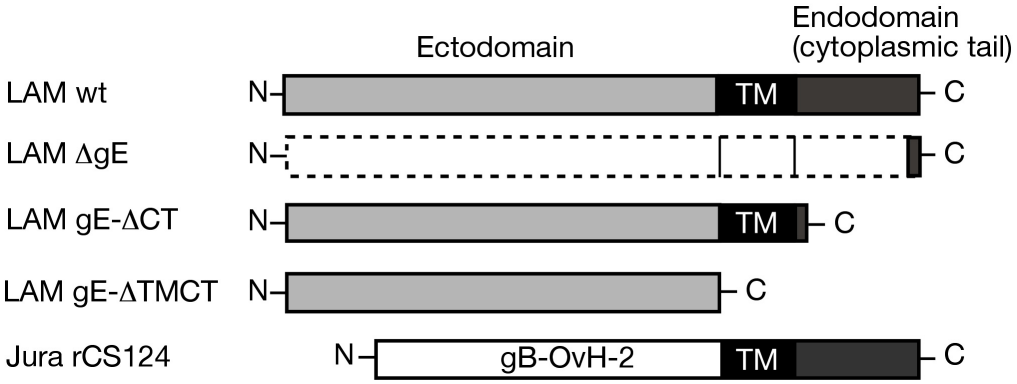

FIG. 1. Schematic drawing of gE deletion mutants and of recombinant virus rCS124.

Infection of Cells

MDBK cells were grown for 1 to 2 days on 30 μm thick sapphire disks with a diameter of 3 mm (Bruegger, Minusio, Switzerland). Sapphires were covered with 8-10 nm carbon obtained by evaporation under high vacuum conditions to enhance cell growth. Then cells were infected with LAM wt or LAM deletion mutants or rCS124 at MOI 1 or 5, kept at 4° C for 1 h to admit adsorption prior to incubation at 37°C for up to 42 h

Freezing of Cells

Cells grown on sapphire disks were frozen in a high pressure freezer (HPM010, BAL-TEC Inc., Balzers, Liechtenstein) as described in detail (Wild, Schraner et al. 2002). The frozen samples were stored in liquid nitrogen until use.

Low Temperature Transmission Electron Microscopy (LTEM)

Frozen cells grown on sapphire disks were transferred into a freeze-substitution unit (FS 7500, Boeckeler Instruments, Tucson, Arizona, USA) precooled to –88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between –30°C and 0°C as described in detail elsewhere (Wild, Schraner et al. 2001) and embedded in Epon. 50 to 60 nm thick sections were analyzed in a TEM (CM12, Philips, Eindhoven, The Netherlands) equipped with a slow scan CCD camera (Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

Determination of Size and Shape of Virions

To determine size and shape distribution of mature virions within the extracellular space 30 images at a final magnification of 89'600 x were recorded from cells infected with LAM wt, ΔgE, gE-ΔCT, gE-ΔTMCT and rCS124 after incubation for 20 h. The diameter of virus particles was measured provided the capsid with its full diameters of about 100 nm was clearly visible. Then the number of viral particles containing 2 capsids and the number of virus particles of oval or irregular shape containing 1 to 3 capsids was counted. Size and shape distribution was expressed as percentage of the total number of viral particles examined.

Statistical Analysis

Data were compared by using the Kruskal_Wallis test for size distribution, and the Dunn's multiple test for group comparison.

RESULTS

The data obtained on BoHV-1 envelopment (Wild, Schraner et al. 2002; Wild, Engels et al. 2005) prompted us to investigate viral proteins possibly involved in controlling the complicated process of envelopment. To illuminate the significance of gE in envelopment we used mutants of which gE, its endodomain or its transmembrane part was deleted or its ectodomain was replaced by gB ectodomain of OvHV-2. Infected cells were immediately arrested by rapid freezing *in situ* at different time periods after infection. High resolution electron microscopy revealed that i) envelopment of all 3 LAM gE deletion mutants and the rCS124 virus followed two distinct pathways identical to those of wt BoHV-1 as described (Wild, Schraner et al. 2002; Wild, Engels et al. 2005), ii) both nuclear and cytoplasmic envelopment was altered, iii) alteration in envelopment enhanced as infection proceeded and iv) the phenotypes of gE-ΔCT, gE-ΔTMCT and of rCS124 did not differ from those of ΔgE. We, hence, document and describe our data with regard to the locus they were observed rather than with regard to the mutant.

RER and Golgi Complex, 8 to 20 h post Infection

Eight to 20 h post infection, capsids of wt virus and of all mutants were found to bud at the inner nuclear membrane resulting in virions comprising capsid, tegument and a thick, dense envelope, located within the perinuclear space and RER cisternae (not shown). Virions with dense envelope were found in the perinuclear space (not shown) and RER cisternae (Fig. 2A) that reached from the outer nuclear membrane close to Golgi fields. At the Golgi complex, virions with distinct spikes were within cisternae at the lateral side which exhibited indications for fission (Fig. 2B) – a process designated packaging (Palade 1975) in the secretory pathway – or they accumulated in Golgi cisternae or Golgi associated vacuoles (Fig. 2C) in large numbers. Virions exhibiting distinct spikes were found within large vacuolar structures (Fig. 2E, F and G) that are assumed either to have derived by fission from Golgi membranes (Fig. 2B), or to represent cross sections through Golgi cisternae. Both viral envelope and membranes of cisternae and vacuoles exhibited distinct spikes (Fig. 2B, E to G). In contrast to wt virus, the gE deletion mutants and rCS124 virus displayed a higher heterogeneity in shape including ovoid and misshaped structures, and virus particles containing more than one capsid (Fig. 2A, C, and G) within Golgi cisternae or Golgi derived vacuoles.

In addition to virions within RER, Golgi cisternae and vacuoles, naked capsids were found to approach Golgi complexes from the cytoplasmic side and were wrapped by Golgi membranes as shown previously (Wild, Schraner et al. 2002; Wild, Engels et al. 2005). The result of such wrapping are virions within a small concentric vacuole, the space between envelope and vacuolar membrane being filled with a dense substance (see Fig. 5E) as seen in wt BoHV-1 infected cells (Wild, Engels et al. 2005). Capsids of LAM wt, ΔgE mutants and rCS124 were observed to bud at membranes of dilated Golgi cisternae, of which membranes were occasionally continuous to the outer nuclear membrane (Fig 2D), and at membranes of vacuoles derived by packaging that already contained virions (Fig. 2E to G). Golgi fields were drastically enlarged in cells infected with LAM wt, LAM ΔgE mutants or rCS124 consisting of up to 7 stacks (Fig. 2A to D).

Size and Shape Distribution of Mature Virions

Virus particles derived by both nuclear envelopment and cytoplasmic envelopment are fully enveloped mature virions. Shape and size of mature virions captured anywhere within the cytoplasm and in the extracellular space were rather homogeneous early in infection with LAM wt virus. Shape of wt virus remained rather sphere-like whereas size of virions varied considerably 40 h after infection (Schraner, Engels et al. 2004) (Fig. 3A). Size and shape, however, varied drastically in all 3 deletion mutants and in rCS124 even at 8 to 20 h of infection (Figs. 3B to F) showing sphere-like, ovoid, or irregularly shaped particles containing one, two or even 5 capsids (Fig. 3E) in a given section plane. Size distribution of viral profiles is displayed in Fig. 4 showing that the diameter of sphere-like particles ranged from 150 to 210 nm with a peak at about 180 nm in LAM wt virus. Diameters of LAM ΔgE, LAM ΔgE-CT, LAM ΔgE-TMCT and rCS124 ranged form about 150 to 340 nm, peaking at 190 to 210 nm. Statistical analysis employing the Kruskal-Wallis and Dunn's multiple comparison test revealed a highly different size distribution ($P < 0.001$) between wt virus and all 4 mutants.

Besides size differences, the number of misshaped virus particles and of particles containing more than one capsid was higher in LAM ΔgE, LAM ΔgE-CT, LAM ΔgE-TMCT and rCS124 than wt virus (Table 1).

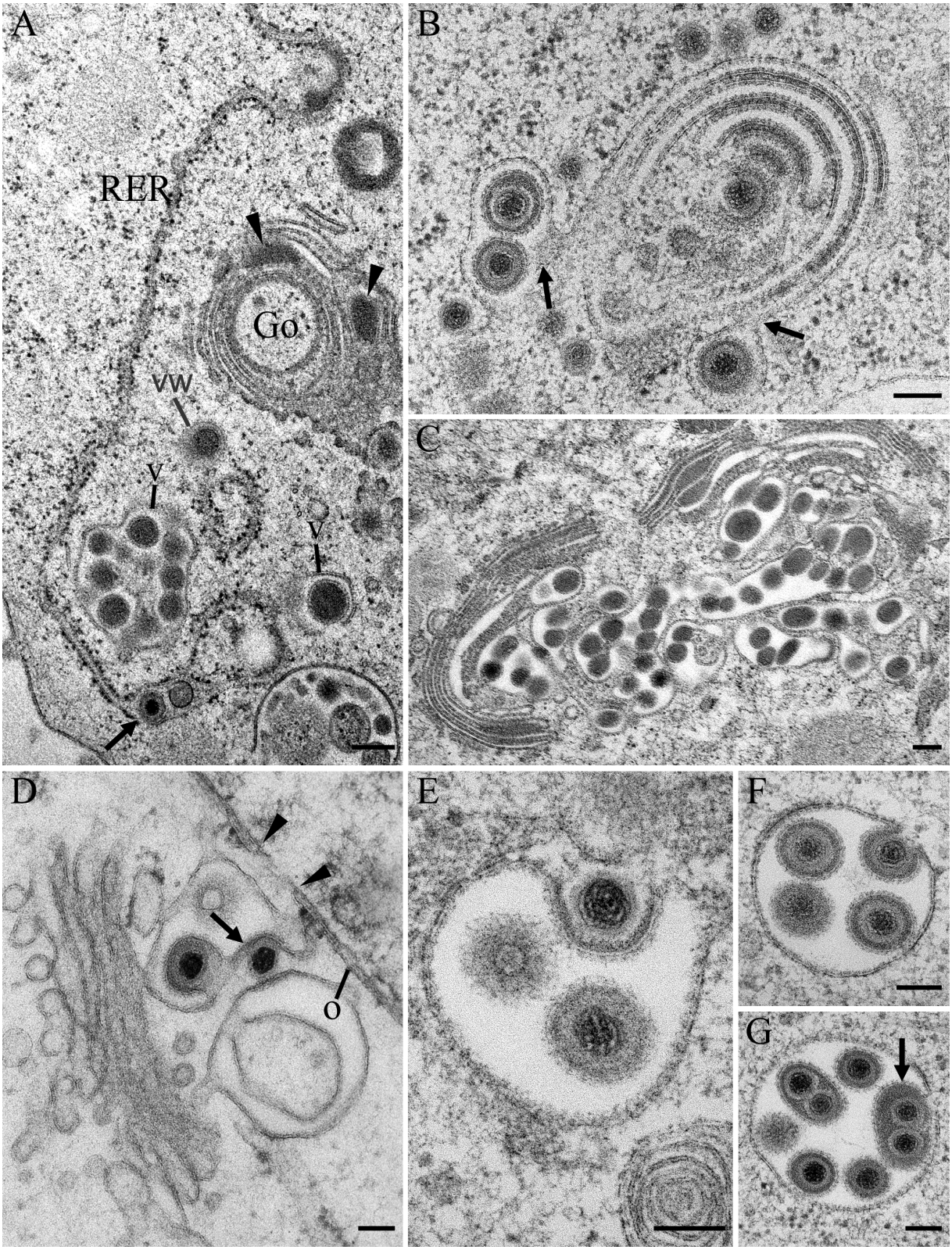

FIG. 2

FIG. 2.

Phenotypes of the nuclear pathway (A, B, C, E to G) and cytoplasmic pathway (B, D to G) in envelopment of LAM ΔgE (A), LAM gE-ΔTMCT (B) LAM gE-ΔCT (C), rCS124 (E to G), and wt virus (D) 16 to 18 h post infection. (A) RER cisterna extending from the nuclear area (top), to the cell periphery contains one (or two) virion (arrow) in close vicinity to Golgi cisternae or vacuoles derived by packaging (v) or wrapping (vw) containing virions. The Golgi complex (Go) engulfs 2 misshaped tangentionally cut virions (arrowheads). (B) Large Golgi complex with two dilated cisternae, which are close before being dispatched (arrows). One contains one virion the other two virions, all of them exhibiting clear spikes. One capsid is partially enveloped in the center of the Golgi complex, and 5 capsids approach Golgi membranes from the cytoplasmic side. (C) Large Golgi complex with dilated cisternae full of tangentionally cut virions many of them of ovoid shape. (D) C-capsids at an early (arrow) and late stage of budding at a Golgi membrane that continues into the outer nuclear membrane (o). The dilated nuclear pore is distinctly bordered (arrowheads). (E to G) Capsids at early (E), intermediate (F) and very late stages (G, arrow) of budding at vacuoles or cross-sectioned Golgi cisternae containing normal virions and two “twins” (G). Bars: 200 nm (A, C); 100 nm (B, D-G).

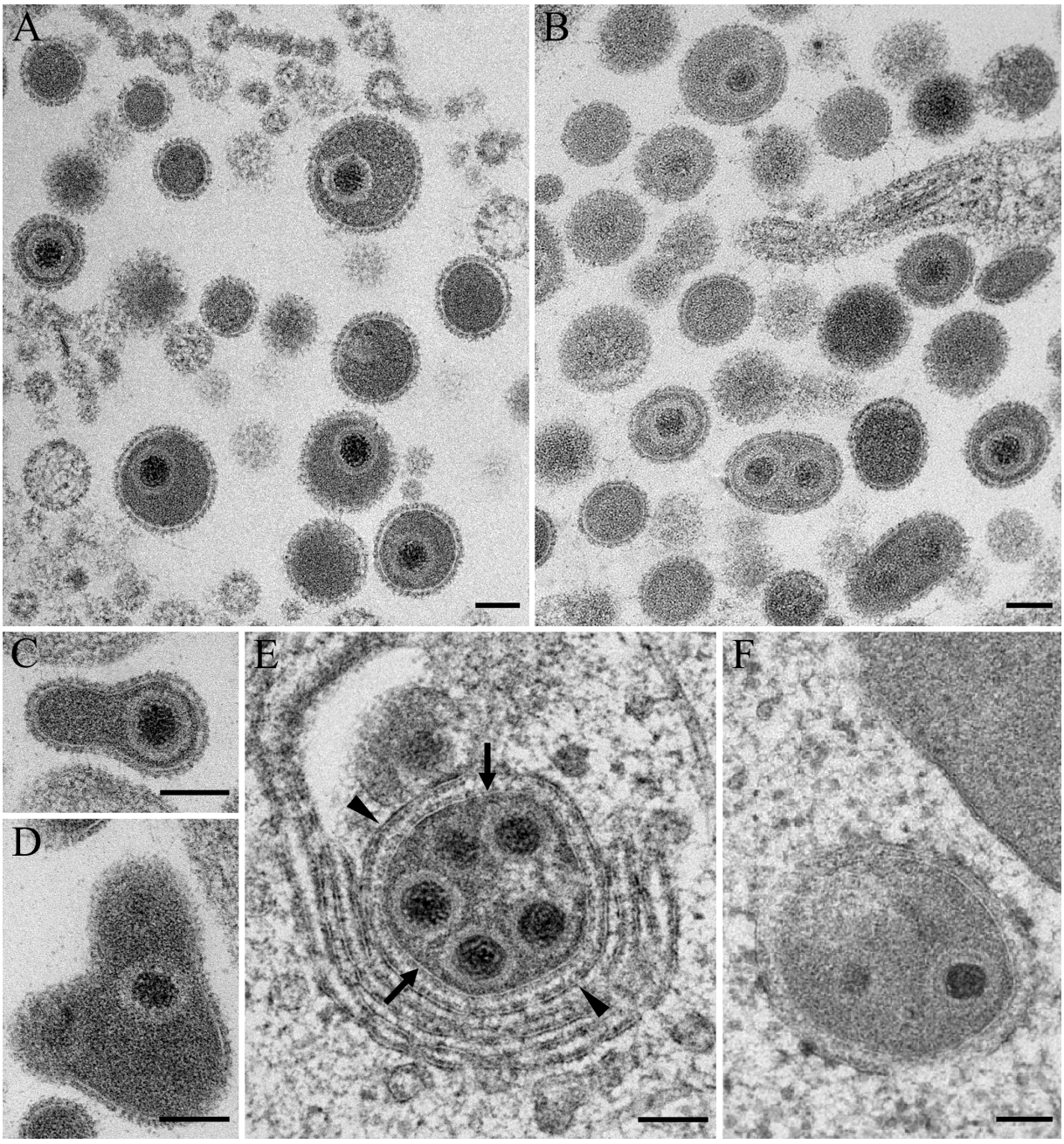

FIG. 3. Variation in size and shape of LAM wt late in infection (A), and of LAM ΔgE (B to D and F) or rCS124 (E) early in infection. (A to D and F) Virions of various sizes and shapes ranging from spheres to ovoids exhibiting distinctly ultrastructural details in the extracellular space of LAM wt 40 h post infection (A), and of LAM ΔgE (B to D) 16 to 22 h post infection. (E) Virus particle with 5 capsids in this section plane with an envelope exhibiting spikes (arrows) surrounded by a Golgi membrane (cisterna or vacuole) exhibiting also spikes (arrowheads). (F) Virus particle with two capsids (one is tangentionally sectioned) within a vacuole the space between envelope and vacuolar membrane being partially filled with a dense substance. Bars: 100 nm.

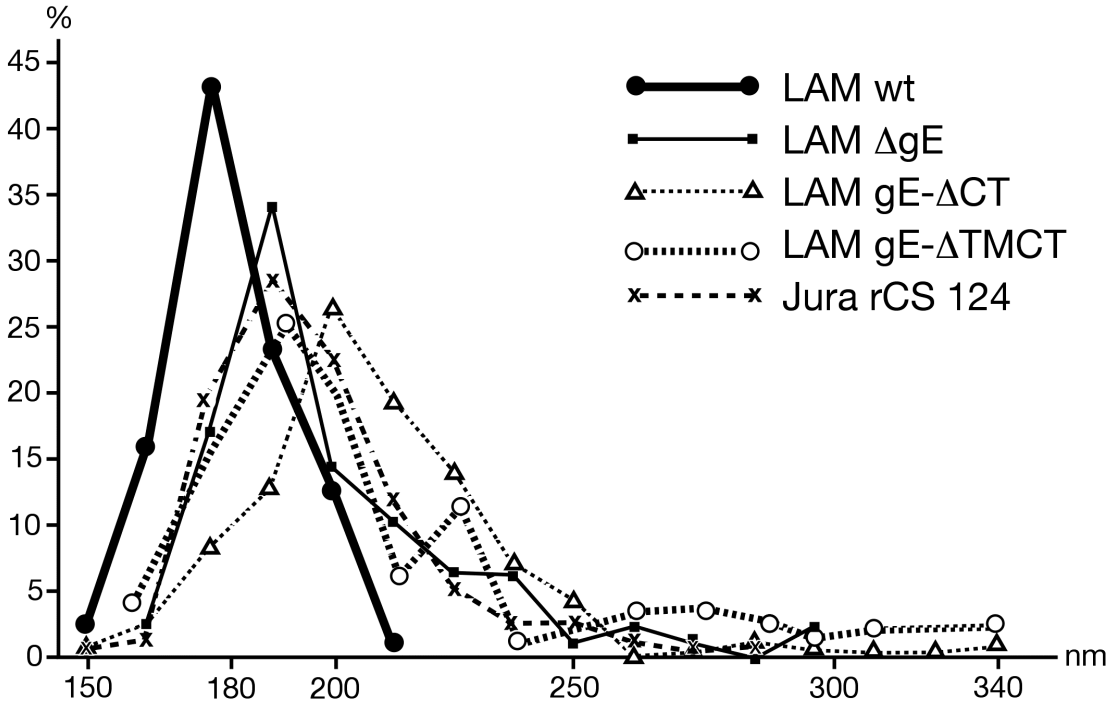

FIG. 4. Size distribution of LAM wt, LAM deletion mutants, and rCS124 in the extracellular space 20 h post infection. Sizes of LAM wt ranged from 150 to 210 nm, of LAM ΔgE from 160 to 310 nm, of LAM gE-ΔCT LAM gE-ΔTMCT from 150 to 340 nm, and of rCS124 from 150 to 300 nm. Data were obtained from sphere-like particles displaying the entire capsid.

Virus	Total number of viral particles	Viral particles with 2 capsids	Misshaped Viral particles
LAM wt	137	0%	7%
LAM ΔgE	319	4%	19%
LAM gE-ΔCT	178	4%	13%
LAM gE-ΔTMCT	239	6%	17%
rCS124	198	5%	13%

TABLE 1. Relative amount of viral particles with 2 capsids, and of misshaped viral particles containing 1 to 3 capsids in a given section plane detected on 30 micrographs taken at random

Nuclear Envelope, 8 to 20 h post Infection

Naked capsids approaching the outer nuclear membrane, Golgi membranes and membranes of vacuoles must gain access to the cytoplasm. As described in detail earlier, the route from the nucleus into the cytoplasm is most likely via impaired nuclear pores (Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005). Nuclear pores of mock infected cells measured 110 to 120 nm in diameter. Nuclei of cells infected with wt virus, deletion mutants or rCS124 had pores of similar sizes (Fig. 5A). In addition, pores, delineated by intact membranes at least at one side, were measured 130 to about 900 nm (Figs. 2D, 5A to C), through which nuclear material and capsids gained access to the cytoplasm (Fig. 5C). Nuclear material and cytoplasmic matrix never merged.

The presence of normal and misshaped virions within Golgi saccules implies that these virions either resulted from budding of capsids into Golgi cisternae or that they reached these cisternae from within the Golgi system. It is considered likely that virions derived by budding at nuclear membranes are transported from the perinuclear space via RER into Golgi cisternae (Leuzinger, Ziegler et al. 2005; Wild, Engels et al. 2005). If this idea were correct alteration of the budding process at nuclear membranes would be expected. Indeed, capsids of the 3 deletion mutants and of rCS124 accumulated to small or large clusters for budding at the inner nuclear membrane (Fig. 5D and E) into the perinuclear space acquiring both tegument and a dense envelope. Budding resulted in virions containing one or more capsids (Fig. 5E). The inner nuclear membrane seems to be invaginated either from one side within the section plane or from underneath or above the section plane (Fig. 5D and E). Small clusters of virions with a dense envelope were found to protrude into the perinuclear space and associated RER cisternae (Fig. 5D) together with nuclear material.

Nuclear Envelope, RER and Golgi Complex, 20 to 40 h post Infection

Heterogeneity in shape and size and in the number of capsids per virus particle was apparently much higher for LAM wt (Fig. 3A), LAM deletion mutants and in rCS124 late in infection (data not shown) as documented previously for LAM wt and LAM ΔgE (Schraner, Engels et al. 2004). To clarify where misshaped virus particles are formed, we investigated cell 20 to 40 h after infection. Capsids were found to aggregate together with small or large amounts of an amorphous substance, presumably tegument proteins, within both the nucleus (Fig. 6A) and the cytoplasm (Figs. 5G, 6A and B). The cytoplasmic capsid-tegument aggregations were often surrounded by 2 distinct membranes in cells infected with any of the LAM deletion mutants or rCS124. The outer membrane was studded with ribosomes. It seems that the inner membrane derived from the inner nuclear membrane during budding of the aggregates from the nucleus into the perinuclear space (Fig. 5F), were finally transported into RER cisternae (Fig. 6A and B). RER (Fig. 5G) and Golgi membranes (Fig. 2B and C) were found to be increased, and nuclear membranes formed coil-like structures bending into the cytoplasm (Fig. 6C) or into the nucleus (Fig. 6D) or they were duplicated (Fig. 6E).

Capsid-tegument aggregates, which consisted of only a few to dozens of capsids, always appeared as solid structures within the nucleus (Figs. 6A). However within the cytoplasm, they were either solid (Figs. 6A and B; 7D) or rather loosely arranged (Fig. 7A to C). The solid capsid-tegument aggregates within the cytoplasm were either bound by two membranes – the outer being studded with ribosomes – or were free within the cytoplasmic matrix (Fig. 7D). The difference in demarcation of these aggregates implies different routes from the site of origin – the nucleus (Fig. 6A) – into the cytoplasm. One route would involve budding at the inner nuclear membrane (Fig. 5F), the other release via impaired nuclear pores as possibly captured in Fig. 7B. The loose capsid-tegument aggregates were associated with cell membranes where capsids were found to bud at Golgi or vacuolar membranes or at RER membranes (Fig. 7C). In addition, accumulation of tegument at Golgi sites was also found to be associated with virus particles (Fig. 7A). Capsid-tegument aggregates were occasionally observed in LAM wt in the nucleus and cytoplasm infected cells 40h post infection. However, the estimated number was less than 1 per 100 cellular profiles whereas the number of capsid-tegument aggregates in all deletion mutants or rCS124 was estimated to be about 5 per 100 cellular profiles at 40 h post infection.

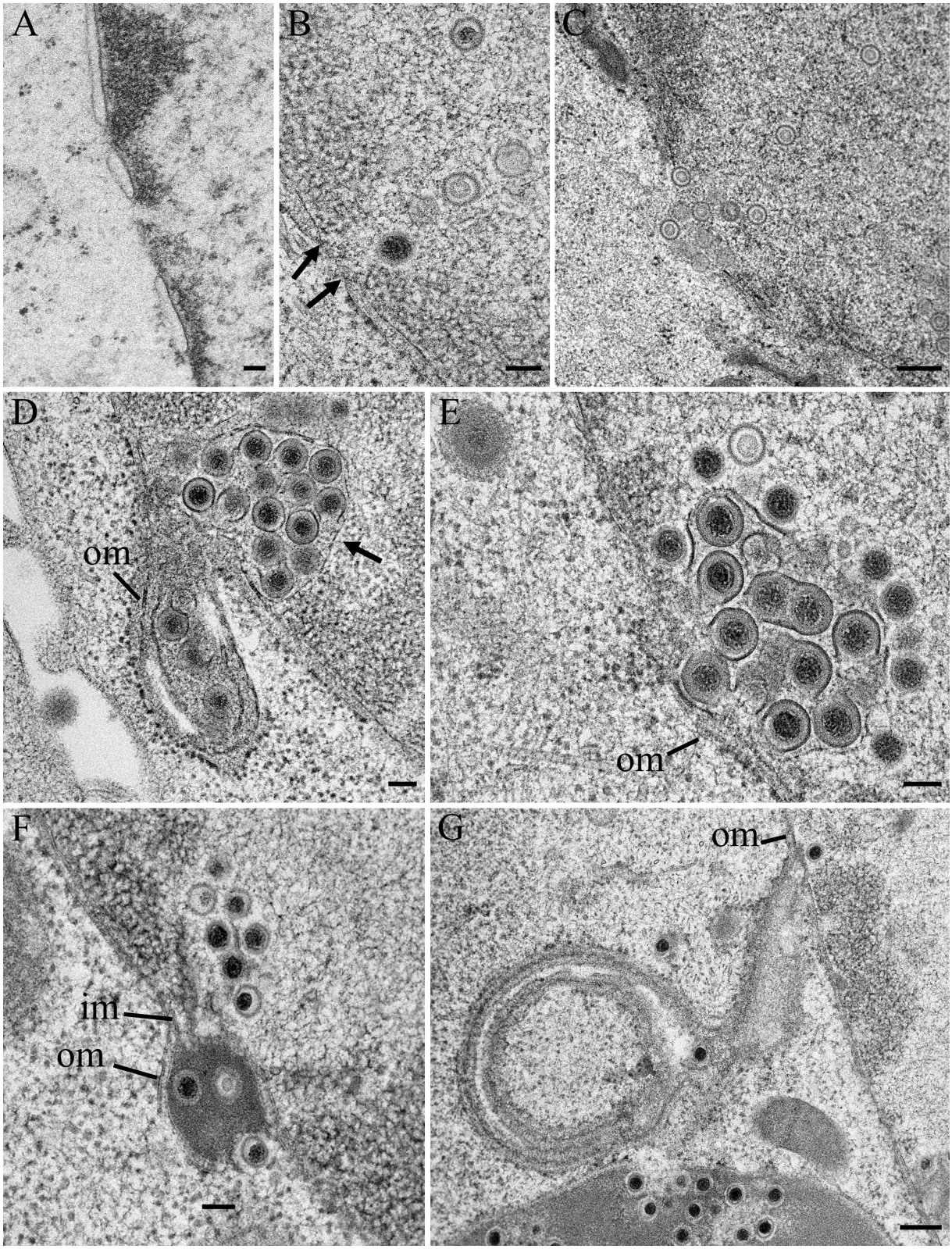

FIG. 5

FIG. 5.

Alteration at the nuclear surface and phenotypes of LAM ΔgE (A, D and G) and rCS124 (B, C, E and F) 10 h (A), 16 h (B, E and F), 21 h (C) or 40 h (D and G) post infection. (A) Normal and dilated nuclear pores clearly defined by nuclear membranes. (B) Nuclear C-capsid (c) in front of a distinctly defined (arrows) nuclear pore slightly larger (130 nm) than the capsid. (C) B-capsids escaping the nucleus via a dilated nuclear pore. Note the nucleoplasm does not merge with the cytoplasm. (D) Accumulation of capsids together with membranous structures (arrow) in front of 3 capsids surrounded by tegument entering the perinuclear space. The inner nuclear membrane (im) delineates the virus-tegument cluster whereas the integrity of the outer nuclear membranes (om) is lost. (E) Cluster of budding capsids at the nuclear periphery acquiring tegument and a dense envelope. (F) Cluster of virions – many are not completely formed – surrounded by a dense membrane (arrow) in front of a nuclear protrusion containing virions. (G) Continuum between perinuclear space and a membranous structure comprising a lumen with a C-capsid, and several layers of tightly packed membranes.. At the bottom is a part of a huge accumulation of capsids and probably tegument bordered by 2 membranes (see Fig 5A). n, nucleus. Bars: 100 nm (A, B, D to F); 300 nm (C, G).

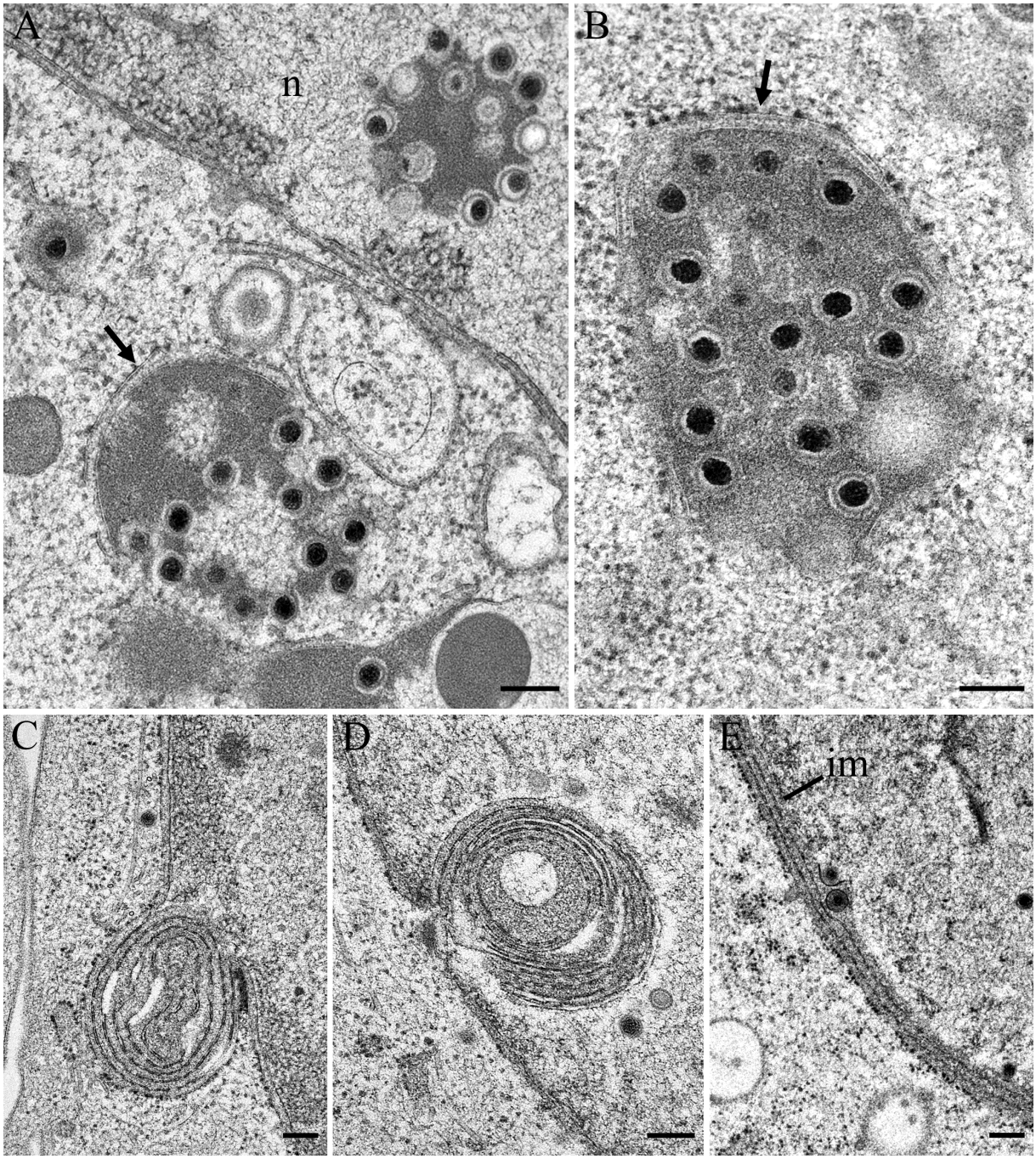

FIG. 6

FIG. 6.

Alterations at the nucleus 20 to 40 h post infection of LAM ΔgE (A and B), rCS124 (C and D) and LAM gE-ΔTMCT (E). (A and B) Clusters consisting of capsids and electron dense substance – most probably tegument – in both nucleus (n) and cytoplasm 40 h post infection. The cytoplasmic clusters are partially (matter of section plane?) enveloped by two membranes; the outer is studded with a few ribosomes (arrows). (C and D) Coils of nuclear membranes protruding into the cytoplasm or nucleus 16 h post infection. (E) Doubled nuclear membranes with capsids at early and very late stages of budding at the inner most membrane (im) 18 h post infection. Bars: 200 nm.

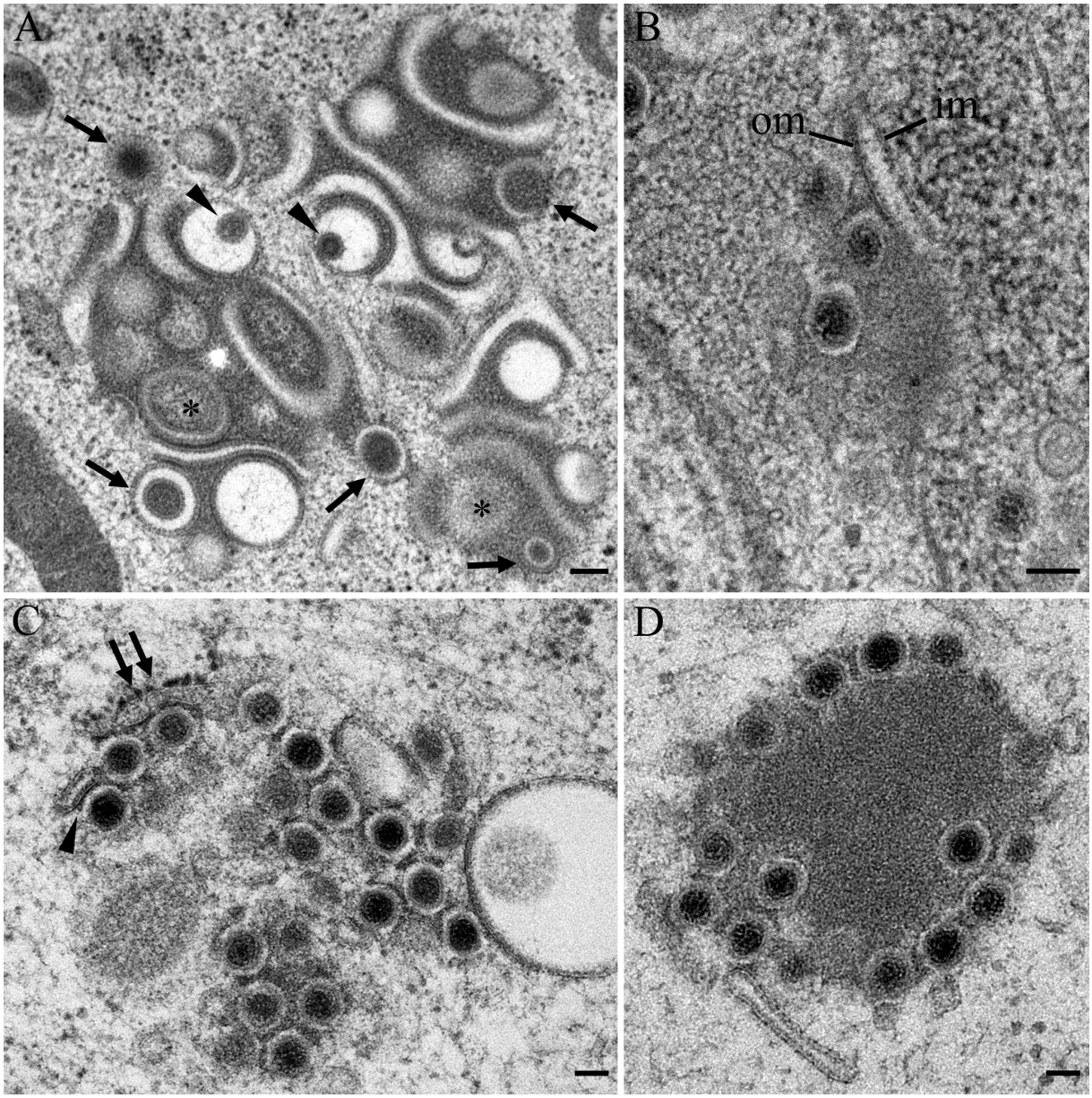

FIG. 7. Cytoplasmic phenotypes of LAM ΔgE (A and B), LAM gE-ΔCT (C) and LAM gE-ΔTMCT (D) 20 to 40 h post infection. (A) Accumulation of tegument-like substance at membranes devoid of ribosomes together with capsids (arrowheads) and virions (arrows), some being in vacuoles 22 h post infection. The ovoid structures (asterisks) might represent misshaped virions. (B) Capsid-tegument cluster associated with the nucleoplasm. The inner nuclear membrane (im) seem to continue into the outer nuclear membrane (om). (C and D) Clusters of capsids together with tegument are free in the cytoplasm. Capsid in early stages of budding at undistinguishable membranes (arrowhead), and at RER membranes (2 arrows). Bars: 200 nm (A); 100 nm (B to D).

DISCUSSION

High resolution electron microcopy of cells infected with LAM gE deletion mutants or rCS124 revealed novel aspects of the significance of gE in envelopment of BoHV-1 (Fig. 8): i) Envelopment of LAM gE deletion mutants and rCS124 followed the same two pathways as wt virus. However, the population of LAM gE deletion mutants and rCS124 was much more heterogeneous considering size and shape and number of capsids per virus particle than that of wt virions. ii) Capsids of gE deletion mutants and rCS124 accumulated for budding at the nuclear periphery as infection proceeded. iii) Virion of LAM gE-ΔCT accumulated within Golgi cisternae. iv) Capsids accumulated together with tegument to solid aggregates within the nucleus. v) Solid capsid-tegument aggregates were present within the perinuclear space and RER cisternae as well as within the cytoplasmic matrix. vi) Capsids and tegument also formed loose aggregates within the cytoplasmic matrix. Capsids at the periphery of loose aggregates initiated budding at cell membranes. vii) Deletion of the entire gE or only of the transmembrane part or of the endodomain, and replacement of the ectodomain by the gB ectodomain of OvHV-2 had similar effects on envelopment. viii) Viral growth of all mutants was only slightly affected late in infection. ix) gE is inserted into nuclear membranes early in infection. x) Spikes, which represent the morphologic substrate of glycoproteins, are inserted into membranes of transport vacuoles exposing the cytoplasmic tail to the cytoplasmic matrix.

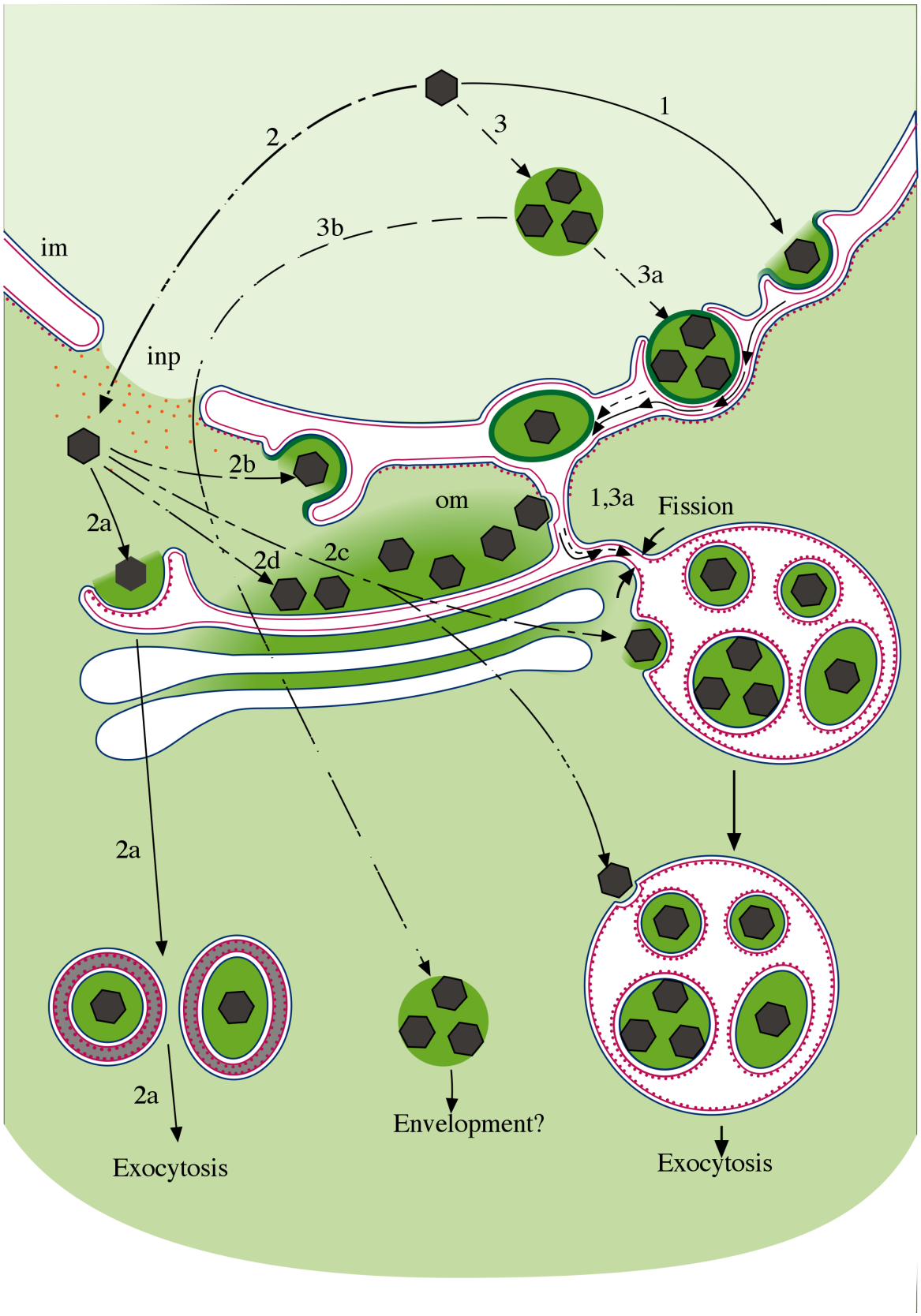
1
2
3
3b
3a
im
inp
2b
2a
om
1,3a
Fission
2d
2c
2a
2a
Exocytosis
Envelopment?
Exocytosis

FIG. 8

FIG. 8.

Schematic drawing of envelopment of BoHV-1 lacking gE or one of its domain. ***Nuclear Envelopment*** (1): Budding of capsids through the inner nuclear membrane results in normal and misshaped virions that are transported from the perinuclear space via RER cisternae into Golgi cisternae for packaging into transport vacuoles of various sizes containing one or more virions. Retardation of fission results in accumulation of virions within Golgi cisternae. After infection proceeds, capsid-tegument aggregate are formed within the nucleus (3) that bud at the inner nuclear membrane (3a). The resulting particles can be transported via RER into Golgi cisternae. ***Cytoplasmic Envelopment*** (2): Capsids exit the nucleus via impaired nuclear pores (inp), approach cell membranes from the cytoplasmic side inducing budding at narrow Golgi cisternae referred to as wrapping (2a), at the outer nuclear membrane (2b), dilated Golgi cisternae (2c) and Golgi derived vacuoles, and RER membranes. Nuclear capsid-tegument aggregates may exit the nucleus via impaired nuclear pores (3b). Capsids and tegument may also aggregate in the vicinity of cell membranes where capsids induce fusion (2d). im = inner nuclear membrane, om = outer nuclear membrane.

There is strong evidence that BoHV-1 aquire the definitive envelope and tegument by budding at the inner nuclear membrane or, after nuclear exit via impaired nuclear pores, by budding at Golgi membranes and outer nuclear membrane (Wild, Schraner et al. 2002; Wild, Engels et al. 2005). The two pathways were also apparent in cells infected with gE deletion mutants. Envelopment was not severely affected early in infection up to about 20 h. However, the population of LAM wt consisted predominantly of sphere-like virions with variation in size from 150 to 210 nm whereas the population was more heterogeneous in gE deletion mutants considering size and shape. Size distribution of sphere-like viral profiles of all mutants was irregular due to larger diameters compared to wt virus. Increased heterogeneity in size and shape of LAM-ΔgE mutants clearly indicate that gE is involved though not essentially in envelope formation and tegument acquisition in the course of budding. If virions of irregular shape and size and number of capsids within RER and Golgi cisternae (Figs. 2C, 3E and F) originated by budding at the inner nuclear membrane followed by intraluminal transportation one action site of gE would be at the inner nuclear membrane. Other action sites of gE would be at all those membranes budding was found to take place i.e. the outer nuclear membrane, and membranes of Golgi cisternae and of vacuoles. Completion of budding is achieved by fission of the envelope from the original membrane at the site the envelope is pulled behind the capsid that becomes concomitantly surrounded by tegument proteins (Wild, Schraner et al. 2002; Wild, Engels et al. 2005). To our knowledge, mechanisms of budding and coordinated acquisition of tegument are not yet understood. The machinery for fission (Peters, Baars et al. 2004) can be assumed to be integrated in cell membranes from which vesicular-like structures are dispatched. Initiation of budding, acquisition of tegument proteins, and the budding process per se are reasonable to assume to be under viral control. Tegument proteins are largely asymmetrically organized except of a small region of tegument-capsid interaction where tegument is icosahedrally ordered (Zhou, Chen et al. 1999). Variation in size and shape may arise because acquisition of tegument proteins and subsequent fission is not optimally controlled. Variation in size and shape was found in LAM wt virus Jura wt virus (Schraner, Engels et al. 2004) and was shown for HSV-1 (Grunewald, Desai et al. 2003). Size and shape variation was remarkably enhanced in gE deletion mutants suggesting gE to be involved in tegument assembly or in budding per se.

Heterogeneity in virion morphology was increased in both wt virus and ΔgE mutants 20 to 40 h post infection (Schraner, Engels et al. 2004) (Fig.3A). In all gE deletion mutants, capsids accumulated at the nuclear periphery for budding (Fig. 5D to F) or capsid-tegument aggregates were formed within the nucleus (Fig. 6A) when infection proceeded indicating enhancement of disturbance in virion formation during budding or prior to budding at the inner nuclear membrane. Capsid-tegument aggregates were found to escape the nucleus via a process similar to budding at the inner nuclear membrane (Fig. 5F) resulting in enveloped solid capsid-tegument aggregate within the perinuclear space from where they were obviously transported into RER cisternae (Fig. 6A and B). Accumulation of budding capsids at the nuclear periphery, and intranuclear formation of capsid-tegument aggregates support the idea that gE is involved during budding at the inner nuclear membrane, and further suggest gE to have a direct or a signaling effects on tegument acquisition at the nuclear level.

In contrast to membrane bound solid capsid-tegument aggregates within RER cisternae, the non delineated solid capsid-tegument aggregates within the cytoplasmic matrix (Fig. 7D) may have been formed within the cytoplasm (see below) or, more likely, originated within the nucleus and gained access to the cytoplasm– as possibly captured in Fig. 7B – via impaired nuclear pores (Fig. 5C). Similar tegument aggregates have also been shown in the cytoplasmic matrix of cells infected with PRV where gE and gI or gM (Brack, Dijkstra et al. 1999), or gM and U_L11 were deleted (Kopp, Granzow et al. 2004). The protein around the capsids was shown to be tegument. In MDBK cells infected with gE deletion mutants, capsids located at the periphery of capsid-tegument aggregates initiated budding whenever they came in close contact with membranes of RER, Golgi complex, and vacuoles. Whether or not budding of such aggregates would be successful if enough membranes were available is unknown. We did not find any indications for successful budding of large aggregates at Golgi membranes.

Besides the solid capsids-tegument aggregates, tegument accumulated together with capsids in the vicinity of Golgi membranes (Fig. 7A) or other membranes (Fig. 7C). These loosely arranged aggregates are considered likely to develop within the cytoplasm because there was no equivalent found within the nucleus, and because they were always associated with cell membranes. For budding at cell membranes both capsid and tegument must be transported to their cytoplasmic side. If the budding process is disturbed viral particles of various size and/or shape containing one or more capsids will be formed provided the amount of membranes is sufficient at a given budding site. Both solid capsids-tegument aggregates and loose

accumulation of tegument and capsids were also found in wt virus infected cells though less numerous than in ΔgE mutants infected cells indicating that gE per se is not responsible for the formation of such aggregates. The lack of gE rather seem to facilitate their formation. If budding does not succeed accumulation of capsids and tegument at the cytoplasmic side of membranes will be expected. Loose capsid tegument aggregates were also found in cells infected with PRV deleted of gE, gI and gM (Brack, Dijkstra et al. 1999) or deleted of gM and UL11 (Kopp, Granzow et al. 2004).

Capsids of all gE deletion mutants were found to bud at dilated Golgi cisternae or vacuoles containing already virions (Fig. 2E to G) in contrast to LAM wt virus and BoHV-1 wt strain Jura (Wild, Engels et al. 2005). Budding into Golgi cisternae or vacuoles were found to take place in HSV-1 (Leuzinger, Ziegler et al. 2005) and cytomegalovirus (Homman-Loudiyi, Hultenby et al. 2003). It is known that the release of virus is retarded in HSV-1 infected cells. The same seems to be true in BoHV-1 lacking gE or one of its component indicating that formation of vacuoles and/or its centrifugal transportation is reduced. Viral transportation from the Golgi complex towards the cell periphery has been shown to depend on the cytoplasmic tail of gE (Tirabassi, Townley et al. 1997; Brack, Klupp et al. 2000; Wisner, Brunetti et al. 2000). Spikes, which represent the morphologic substrate of glycoproteins, are inserted into vacuolar membranes (Fig. 2E-G) and into Golgi membranes (Fig. 3E) prior to fission (Fig. 6D in ref. (Wild, Engels et al. 2005). Thus the functional domain in vacuolar transportation can only be the cytoplasmic tail (Fig. 9). However, accumulation of virions within Golgi cisternae found in any of the gE deletion mutants indicates that formation of vacuoles that involves fission is retarded. Fission of vacuoles from Golgi membranes probably requires a similar machinery as fission of virions from Golgi membranes or from nuclear membranes in the course of budding. Fission of vacuoles is – in contrast to budding – not accompanied by tegument acquisition. Thus one may argue that gE effects fission per se.

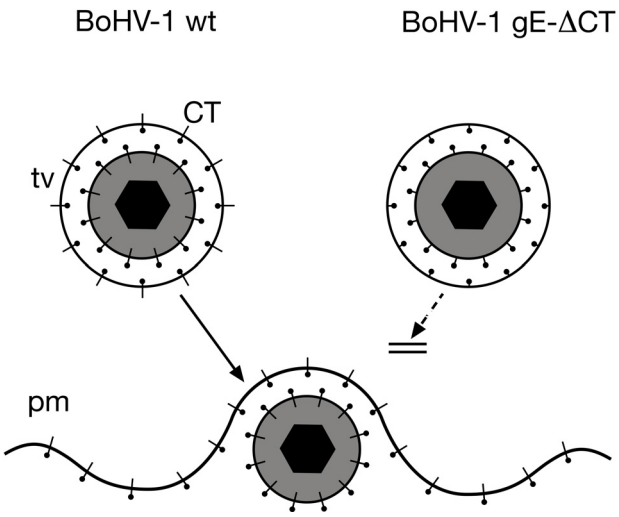

FIG. 9. Glycoproteins are inserted into Golgi membranes. Consequently transport vacuoles (tv) derived by fission from Golgi membranes contain glycoproteins readily seen as spikes in Figs. 2E to G. The cytoplasmic tail (CT) faces the cytoplasm. This is the only site gE can function as a pilot protein to direct vacuoles toward the plasma membrane (pm).

gE is embedded into the viral envelope. Its cytoplasmic tail is located at the inner side of the envelope exposed to the tegument. We speculated that gE is responsible for correct acquisition of tegument proteins leading to sphere-like virus particles of diameters of 200 nm as revealed by cryo-microscopy (Zhou, Chen et al. 1999; Grunewald, Desai et al. 2003). If this assumption was correct, gE mutants with only the cytoplasmic tail deleted would have identical effects as LAM-ΔgE. Indeed, the observations we made in cells infected with LAM gE-ΔCT and LAM gE-ΔTMCT mutants were similar to those in cells infected with LAM-ΔgE mutants confirming the idea that gE acts as a regulator in tegument protein deposition concomitantly to budding of capsids at nuclear membranes or Golgi membranes. To further prove this idea we used a recombinant virus in which the gE ectodomain was replaced by the gB ectodomain of OvHV-2. Surprisingly, the results were very similar to those obtained in cells infected with any of the LAM deletion mutants indicating that the entire gE is involved in the process of optimal tegument acquisition and envelope formation.

To play its role in the budding process, gE localizes in cell membranes where budding of capsids take place. Immunolabeling revealed that gE was localized on the entire RER which includes nuclear membranes (Rychlowski, Rijsewijk et al. 2001). Indeed, confocal microscopy of cells infected with LAM wt also clearly demonstrated that gE is integrated into nuclear membranes. These findings do not only confirm that gE can act at the nuclear level, they also imply that virions formed by budding at the nuclear membrane must contain gE in their envelope. The presence of various glycoproteins in nuclear membranes were also demonstrated in many members of the herpesvirus family (Cranage, Smith et al. 1988; Gilbert, Ghosh et al. 1994; Hutchinson, Roop-Beauchamp et al. 1995; Browne, Bell et al. 1996; Lee and Longnecker 1997; Pertel, Spear et al. 1998; Oravcova, Kudelova et al. 2000). Consequently, these glycoproteins will become part of the viral envelope as the result of budding. The reason why they cannot be dedected by immunolabeling in virions within the perinuclear space and associated RER cisternae on the electron microscopic level is discussed in detail elsewhere (Stannard, Himmelhoch et al. 1996). The envelope of virions within the perinuclear space and RER cisternae contains a dense substance that was considered likely to consists of antifusion proteins for preventing the envelope from fusion with the membranes of those compartments virions are transported through (Wild, Schraner et al. 2002; Wild, Engels et al. 2005), or for facilitating intraluminal transportation. There is strong evidence that virions are transported from the perinuclear space into Golgi cisternae where these proteins are possibly cleared (Wild, Schraner et al. 2002). Intraluminal transportation of virions with inserted glycoproteins is supported by the demonstration of mannose in virions within the perinuclear space (Poliquin, Levine et al. 1985). Consequently, virions carrying glycoproteins derived by budding at nuclear membranes carry them to Golgi cisternae. As shown in Fig. 2 virions containing spikes (Figs. 2E to G, 3E) – the morphologic equivalent of glycoproteins – may accumulate within Golgi cisternae suggesting that glycoproteins are accessible for immunostaining. This is readily demonstrated by immunofluorescence microscopy using antibodies against gE/gI of LAM wt virus (not shown). Using the same antibody to detect LAM gE-ΔCT accumulation of signals at Golgi regions was reduced. Instead, the nuclear rim was intensely labeled indicating that transportation of gE/gI towards the Golgi region was reduced. Of course, glycoproteins must also be transported from the RER, the site of synthesis, to the Golgi membranes where they become part of the viral envelope as a result of wrapping by Golgi membranes, or as a result of budding at Golgi derived vacuoles.

The primary site for capsids to acquire an envelope and tegument proteins is the inner nuclear membrane (Granzow, Klupp et al. 2004). Hence, the primary site of gE to be involved in viral formation is the nuclear membrane. gE was shown to act in concert with other glycoproteins such as gI and gD (Farnsworth, Goldsmith et al. 2003), or gI and gM (Brack, Klupp et al. 2000). The presence of gE on the nuclear surface and that of gI and gD (Stannard, Himmelhoch et al. 1996; Wang, Gershon et al. 2001) suggest that these glycoproteins do not only act at the Golgi site (Wang, Gershon et al. 2001; Farnsworth, Goldsmith et al. 2003) but also at the nuclear periphery. Glycoprotein M was reported to act in concert with the tegument protein U_L11 (Kopp, Granzow et al. 2004) which is also located at nuclear membranes (Baines, Jacob et al. 1995) suggesting that these proteins are also involved in viral formation in the course of budding at nuclear membranes as was clearly shown for U_L31, U_L34 and U_S3 in herpes simplex virus 1 (Reynolds, Ryckman et al. 2001; Reynolds, Wills et al. 2002).

Budding requires large amounts of membranes which is possibly provided by de novo synthesis. Capsids of LAM-ΔgE mutants aggregated in large clusters that may overcome the nucleocytoplasmic barrier via a process similar to budding resulting in an enveloped capsids-tegument aggregate within the perinuclear space. The surface area of such aggregates is much smaller than the total surface area of all virions would be if every single budding capsid was acquiring an envelope. LAM-ΔgE mutants thus need less nuclear membrane constituents than wt virus. Consequently, the excess of membranes formed structures like duplication of nuclear membranes and membrane coils. Folding and duplication of nuclear membranes, however, occur also in cells infected with wt HSV-1 (Roizman 2001).

In conclusion, the three most important findings of this study are i) the subtle alterations in viral shape implying gE to act in tegument acquisition and formation of optimally sized virus particles, ii) accumulation of virions within Golgi cisternae suggesting gE to have an effect on fission, and iii) the insertion of spikes in transport vacuoles suggesting that the cytoplasmic tail of gE may have pilot function in vacuolar transportation towards the cell periphery. Capsid-tegument aggregates located at various sites of the envelopmental pathways indicate that gE is involved in both nuclear and cytoplasmic envelopment. The capsids-tegument aggregates are not unique for BoHV-1 lacking gE. Polymorphism and formation of capsid-tegument aggregates enhance when infection proceeds. Formation of single well sized virions is thus critical without the coordinating function of gE when hundreds of capsids need to acquire a proper shell of tegument proteins and an envelope within a short period of time.

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